Kinetics and Mechanisms of the Griess Reaction

Jay B. Fox, Jr.

Eastern Regional Research Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118

The kind and position of substituents on the aniline and naphthylamine reagents used in the Griess method of nitrite analysis were assayed, and the factors that control the rate, amount, and stability of the pigment formed from the reaction were defined. This work has shown that the Hammett relationship may be used to determine in part the utility of the aniline derivatives under the unique conditions of nitrite analysis. However, specific reagent combinations result in poor pigment production because of multiple reactions, multiple products, incomplete conversion, and pigment instability. Impediments to complete conversion of nitrite to diazo pigment include multiple nitrous acid reactions with both the aniline and naphthylamine derivatives and instability of reaction intermediates. Other factors critical to pigment production, including pH, temperature, and concentration of reagents, both relative and absolute, were quantitated. Added reductants usually result in lessened pigment production, except with certain reagent combinations where the same or higher concentrations are produced. Criteria are given for establishing the utility of reagent combinations to be used for nitrite analysis.

The formation of pigments from various nitrosatable compounds (principally aniline derivatives), nitrous acid, and various coupling reagents (principally naphthalene derivatives) was first described by Johann Peter Griess in 1864 (*1*). This "Griess reaction", which forms the diazo pigments of major importance to the dye stuff industry, has been studied extensively. The reaction is of equal utility as a measure of nitrite, as Griess first demonstrated in 1879 (*2*), and is extensively used for this purpose for analysis of nitrite in foodstuffs. There is a major difference in the way the reaction is carried out for the two purposes. The reaction proceeds in three steps: nitrosation, diazonium ion formation, and coupling. The first step is an acid reaction, the second an internal rearrangement, and the third a reaction that proceeds at different rates depending on pH. In the production of dyestuffs, the diazonium salt is prepared from a nitrosated species (NS) in acid solution with excess nitrous acid, crystallized, and then reacted with the coupling reagent (CR) at the optimal pH, usually close to neutrality (*3*). For nitrite analysis, the reaction is carried out at a pH that is a compromise between the optimal pH values for the two pH-dependent reactions, with all three reaction steps continuing simultaneously at limiting nitrite concentrations.

For testing foods, especially meat products, nitrite analysis requires some sample preparation before the Griess reagents are added. In our laboratory, a study of a number of the sample preparation procedures in use globally (*4*) led us to conclude that the amount of nitrite measured depends not only on how much "free" or "bound" nitrite is originally present in cured meats, but also on how the sample preparation procedure affects other compounds that interfere in the color developing reaction. We used the same colorimetric reagents so that we could make direct comparisons of the effect of the various preparation procedures. There are no studies on the effect of different colorimetric reagents on the same preparation procedure. Sawicki et al. (*5*) studied 52 different techniques for nitrite analysis, including almost as many different reagents, but the effectiveness of individual reagents in a given system cannot be evaluated from their data since they also varied preparation procedures. The same is true for almost all the rest of the reported work on nitrite analytical procedures, and no direct systematic comparison of the relative effectiveness of a variety of Griess reagent combinations has been made, nor is one possible from the literature. To evaluate the effect of residual reactants on the Griess reaction, one must determine the critical reaction parameters by systematically studying an analogous series of reactants under varying conditions.

Some optimal operating conditions have been established. Ilosvay (*6*) first recommended the use of acetic acid which results in reaction solution pH values in the range of maximal conversion, pH 2.5–3.0 (*7*), for the sulfanilic acid/1-naphthylamine reactions, but whether this is a universal range is not well established. The NS must be in at least 100-fold molar excess over nitrite for maximal pigment production (*8*, *9*). It has long been known that with a large excess of nitrite only yellow colors are produced (*10*), but the effect of large excesses of reagents has not been studied, nor have the optimal reagent concentrations been established. Incomplete color development may occur if nitrite reacts with the CR. The nitrosation of the coupling reagent, 1-naphthylamine, is the basis for a method of nitrite determination (*11*).

Reductants, particularly ascorbate, reduce diazonium salts (*12*), but neither this nor the previous reaction has been quantitated with respect to a variety of possible NSs and/or CRs. It would be a monumental task to examine even a modest number of NSs and CRs in all possible combinations under all likely reaction conditions. We therefore chose an analogous series of both NSs and CRs to be reacted in a standard set of conditions and then picked reagent combinations representative of a range of reactivities for further study under special conditions.

EXPERIMENTAL

The principal reagents used in this study are summarized in Table I. All reagents were highest purity available, but grade and quality varied. Some reactants were recrystallized when it seemed necessary; however, except for 1-NA and NED (see Table I for definitions of abbreviations), the process made little difference in the rate or amount of conversion. Although MAA and SAN from the two different sources were of different qualities, the rates and amounts of pigment formed were the same. Reagent deterioration was observed for the more reactive reagents, but the only effect appeared to be slower rates or lessened conversions due to a loss of reagent.

For determining the rate constants and total conversion figures the standard reaction system approximated that used for the Association of Official Analytical Chemists (AOAC) colorimetric method (*13*). Concentrations used were 1.0 mM NS, 0.2 mM CR, and 10 μM NO_2^-, 1.5% acetic acid. This concentration of acetic acid is that obtained when the Griess reagents are diluted 1→10 as suggested by Fiddler (*13*) and resulted in a solution pH ranging between 2.75–2.85; over this range the reaction showed negligible variation (*7*). For testing the effect of reductants, concentrations in the reacting solutions were $^1/_{100}$ of the concentrations at which the compounds would normally be added to or found in cured

Table I. Nitrosatable Species and Coupling Reagents Used for the Study of the Griess Reaction, with Abbreviations, Sources, and Purities

reagent	abbr.	source[a]	purity	further treatment
nitrosatable species	NS			
p-nitroaniline	PNA	Eastman	98%	
sulfanilamide	SAN	Baker/Eastman	164–166 °C mp	
sulfanilic acid	SAA	Fisher	Certified/Anal.	
metanilic acid	MAA	City/Eastman	98%	
p-aminobenzoic acid	PABA	Eastman	98%	
p-chloroaniline	PCA	City	70–72 °C mp	
aniline	ANI			redistilled 184–185 bp
p-anisidine		Baker	58–60 °C mp	
p-phenylenediamine		City	139–141 mp	
o-toluidine		Chem. Serv.	A/B	
m-toluidine		Chem. Serv.	A/B	
p-toluidine		Chem. Serv.	A/B	
coupling reagents	CR			
1-naphthylamine[c]	1-NA	City/Eastman	49–50 °C mp	recrystallized
N-1-naphthyleneethylenediamine	NED	MC/B	98%	recrystallized
1-amino-2-naphthalenesulfonic acid	2SN[b]	Eastman	98%	
5-amino-1-naphthalenesulfonic acid	5SN[b]	City	Tech.	recrystallized
5-amino-2-naphthalenesulfonic acid	6SN[b]	City	Tech.	
8-amino-2-naphthalenesulfonic acid	7SN[b]	City	82.8%	recrystallized
8-amino-1-naphthalenesulfonic acid	8SN[b]	City	Tech.	
5-hydroxy-1-naphthylamine	5ON[b]	City	Tech.	
7-hydroxy-1-naphthylamine	7ON[b]	Aldrich	204–207 °C mp	
1,5-diaminonaphthalene	5AN[b]	City	--	
5-nitro-1-naphthylamine	5NN[b]	Chem. Serv.	--	
2-naphthylamine[c]		K & K	--	
6-amino-2-naphthalenesulfonic acid		City	Tech.	
1-methylnaphthalene		City	120–1 °C/17 mm. Hg, bp	
1-naphthol		Fisher	94.4–95.4 °C mp	
N-1-naphthaleneacetic acid		City	130–132 °C mp	
N-1-naphthaleneacetamide		City	159–160 °C mp	
4-amino-1-naphthalenesulfonic acid		City	Tech.	

[a] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned. [b] In order to make the comparison of the effect of position of the substituents easier, the abbreviations refer to the compounds as if they were 1-naphthylamine derivatives. [c] These compounds have been identified as Class one carcinogens by the OSHA (ref. 24).

meats since the normal dilution of meats is 1:100. The concentrations were 25 μM ascorbate, 200 μM cysteine, and 10 μM reduced nicotinamide adenine dinucleotide (NADH).

For these kinetic studies, temperature control had to be within ±0.1 °C and reagent solutions had to be mixed quickly and thoroughly. The former was accomplished by use of a pre-reaction equilibration bath for the reagents and a thermostated optical cell for the reacting solutions, both of which were monitored by thermistor units, which in turn controlled pumps circulating cold water through the bath and cell. Reagents were made up to twice the desired final concentration in two separate solutions; each solution was drawn up into one of two syringes of a dual syringe unit which was then placed in the equilibration bath. To start the reaction, a T-bar plunger depressed both syringe plungers to the same volume, and tubes from the syringes were swaged into a "Y" fitting, the third member of which was a long blunt needle (Figure 1). The latter was inserted into the optical cell through a hole in the sample compartment lid, and the reaction was started by quickly depressing the T-bar plunger. Mixing was complete after passage through the "Y" and needle and agitation of the solution in the cell during filling. The total time for cell filling was about 0.04 min, a negligible time period with respect to all but the fastest reactions. Operation of the dual syringe unit was fast, highly reproducible, and versatile. Pre-reaction experiments, such as those for determining maximal coupling rates by pre-reacting the NS and nitrite, were performed by premixing the desired reagents in tubes in the equilibration bath. At appropriate times, equal volumes of pre-reaction and CR solutions were drawn up and mixed as described to start the reaction.

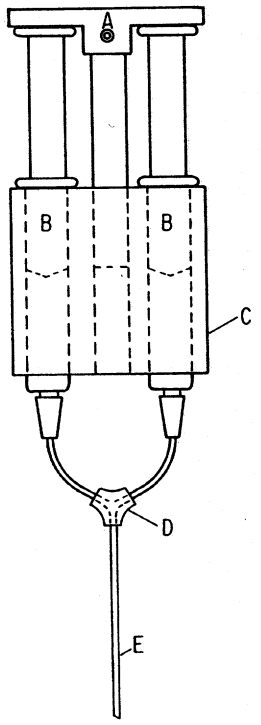

Figure 1. Dual syringe unit. A, T-bar plunger. B, B, 5-mL glass syringe with rubber tipped plungers. C, Brass body, made of solid material to reduce thermal change. D, Y-fitting. E, 18-g stainless steel needle

Mechanisms. The various reaction sequences that contributed significantly to the kinetics observed in this study are summarized in Figure 2. Reactions A and B are the two separately observable reaction steps, formation of the diazonium ion from an aniline derivative and coupling of the ion to a naphthylamine derivative. The first reaction consists of two reaction steps, nitrosation by a nitrosating reagent followed by an internal reaction to form the diazonium ion. The reaction sequence is therefore bimolecular–monomolecular–bimolecular, but in practice the reaction is first order with respect to nitrite because: (a) the colorimetric

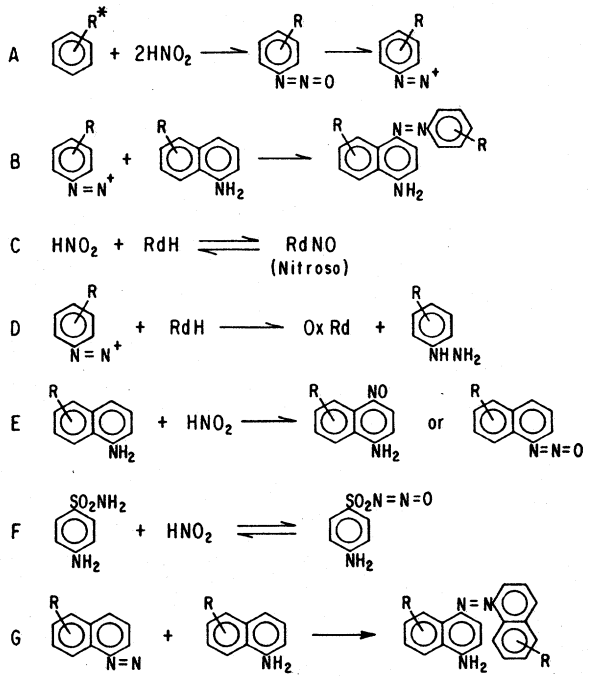

Figure 2. Reaction sequences critical to the formation of the Griess reaction pigment. R = $-SO_2H$, SO_2NH_2, Cl, NH_2OH and any combinations thereof. Rd = reductant

reagents are present in large excess and (b) one of the reaction steps is usually faster than the rest.

In its simplest form, the reaction sequence is:

$$N \xrightarrow{k_1} D \xrightarrow{k_2} G \tag{1}$$

N = [nitrite], D = [diazonium ion], and G = [Griess pigment]. The rate expression is:

$$\frac{dG}{dt} = \frac{N_0 k_1 k_2}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \tag{2}$$

which integrates to:

$$G_t = N_0\left(1 + \frac{k_1 e^{-k_2 t} - k_2 e^{-k_1 t}}{k_2 - k_1}\right) \tag{3}$$

Equation 3 is solvable by computer techniques, but the rate constants may be determined by operational procedures. When either $k_1 \gg k_2$ or $k_1 \ll k_2$ the equation simplifies to a first-order expression for product, dependent on the smaller k value, for example, if $k_1 \ll k_2$;

$$\frac{dG}{dt} = N_0 k_1 e^{-k_1 t} \tag{4}$$

The range of relative rate values over which Equation 4 does not hold is quite narrow. A series of rate curves was calculated with $k_1 = 1$ and k_2 varying from 0.1 to 3.0. The first-order rate constant (k_{obsd}) was calculated from the linear portion for each curve and plotted as a function of $R = k_2/k_1$ (Figure 3). $k_{obsd} = k_2$ to within 2% up to $k_2 = 0.5$; $k_{obsd} = k_1$ to within 2% from $k_2 = 2.0$ and higher. That is to say, only in the range $0.5 < k_2 < 2.0$ would Equation 3 be any different from the one-step reaction expression, and then only if differences of at least 2% could be detected. As a close approximation, the curve of Figure 3 may be used to correct the observed rate constants. The observed rate constant, R_{obsd}, is divided by k_1 to normalize it to the ordinate. The corresponding R value from the curve may then be used to calculate k_2 ($k_2 = Rk_1$). Operationally, the effect of rate limiting by the first step may be eliminated by pre-reacting the NS with nitrite to form the diazonium ion. The coupling reaction is then followed directly. Where R_{obsd} was close to k_1, we applied the procedure to determine k_2. Conversely, the maximal k_1 values were determined from reactions where $k_2 \gg k_1$.

Deviations from first-order curves were invariably due to other causes such as interfering reactions or multiple or side reactions

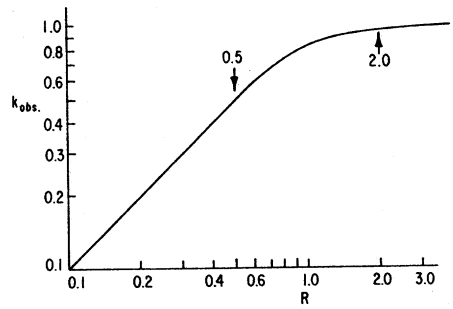

Figure 3. Dependence of the observed rate constant for a two-step reaction on the ratio ($R = k_2/k_1$) of the rate constants for the individual steps. $A \xrightarrow{k_1} B \xrightarrow{k_2} C$ below $R = 0.5$, $k_{obsd} = k_2$; above $R = 2$, $k_{obsd} = k_1$

of the reagents, of which the most important are shown in Figure 2. Of the listed reactions, the most common were those of the type of C, E, or F, which result in a loss or reversible binding of nitrite, and D, which results in a loss of the diazonium ion. The reaction sequences for these two types of interfering reactions may be written:

$$\begin{array}{l} N \xrightarrow{k_1} D \xrightarrow{k_2} G \\ \quad\downarrow k_3 \\ \quad O \end{array} \tag{5a}$$

or

$$\begin{array}{l} N \xrightarrow{k_1} D \xrightarrow{k_2} G \\ \quad\uparrow\downarrow \\ \quad O \end{array} \tag{5b}$$

$$\begin{array}{l} N \xrightarrow{k_1} D \xrightarrow{k_2} G \\ \qquad\searrow^{k_3} \\ \qquad O \end{array} \tag{6}$$

where O = [colorless or unreactive product].

The true rate expressions may be written for these reactions, but in either case the calculation of the rate of the interfering reactions in 5a and 6 may be simplified. Integrating the rate expressions and setting $t = \infty$, that is, completion of the reaction, one finds that the amount of Griess pigment formed is proportional to the ratio of the rate of its formation to the sum of the competing reaction rates, thus:

$$G_\infty = \frac{N_0 k_1}{k_1 + k_3} \tag{7a}$$

$$G_\infty = \frac{N_0 k_2}{k_2 + k_3} \tag{7b}$$

where 7a is for Equation 5a and 7b for Equation 6. For Equation 5b, $G_\infty = N_0$, but where such an equilibrium occurred, the observed rate curves were biphasic, with the rate of the second part of the curve being governed by the rate of reversion of the equilibrium product back to initial reactant. (See "Results".)

One may analyze first-order reactions without knowing either the initial concentration of reactant or the final concentration of product. If three consecutive values of reactant or product are known with equal time periods between the values, both the rate constant and the initial or final concentrations may be calculated (*14*):

$$k_{1_{st}} = \ln \frac{X_2 - X_1}{X_3 - X_2} \Big/ \Delta t \tag{8}$$

$$a_{0 \text{ or } \infty} = \frac{X_2^2 - X_1 X_3}{2X_2 - X_1 - X_3} \tag{9}$$

where X_n is the absolute value of the concentration of reactant. In practice, several different time periods were used to calculate a set of first-order rate constants. If they did not vary with time, it could be assumed that the reaction was indeed first order, and the concentration of reactant or the theoretical final absorption

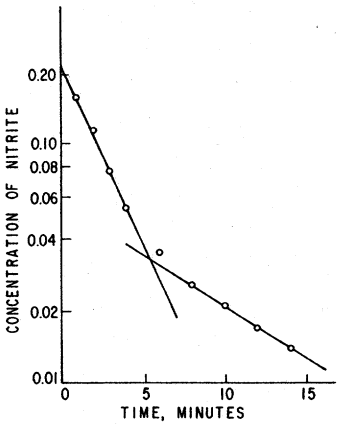

Figure 4. Disappearance of reactant in a first-order reaction where part of the reactant is rapidly converted to an unreactive or uncolored form which subsequently reacts to yield either reactant or product. Biphasic reaction is typical of either the sulfanilamide reaction or reaction in the presence of another nitrosatable compound, for example, ascorbate

values were calculated. The latter values were used when there was uncertainty as to whether or not the reaction was complete.

RESULTS

Rate Constants and Total Absorbance. Table II shows the values for the two calculated first-order rate constants, k_1 and k_2, and Table III the final absorbance at the indicated wavelength of maximum absorbance.

The pigment forming reactions for all NS were first order throughout the entire reaction except for SAN which was biphasic. The initial phase of the reaction with SAN was swift and first order, terminating in a second slow phase which was also first order (Figure 4). With increasing periods of prereaction time, the slow phase started with decreasing quantities of Griess chromophore produced, i.e., some other product, C, was being formed which subsequently decomposed to yield pigment either directly:

$$\begin{array}{l} NS + N_2O_3 \rightarrow D \rightarrow G \\ \quad\downarrow \qquad\qquad\quad \uparrow \\ \quad NS - NO \rightarrow C \end{array} \qquad (10)$$

or by reforming the NS (Equation 5b).

In either case, the evidence strongly indicates reaction with the sulfonamide group. The side reaction must take place with either this group or the benzene nucleus. The sulfonic and sulfonamide groups are meta directing of about equal influence (less than a twofold difference in the Hammett σ values of the two). Under the conditions studied, a biphasic reaction due to ring substitution would have been observed for both. Since the sulfanilic acid reaction was not biphasic, we deduce the formation of a nitroso sulfonamide group in sulfanilamide (F in Figure 2). The nitrososulfonamide group underwent hydrolysis only to the initial reactants, since the reaction resulted eventually in total conversion to the diazo dye. The hydrolysis was first order, with a rate constant of approximately 0.13 min^{-1}.

Ring Substituents: NS. In addition to the compounds shown in Tables II and III, we also tested anisidine, 1,4-benzenediamine and *o*, *m*, and *p*-toluidine as NS, but none of these coupled to form a pigment. When nitrite was prereacted with these noncouplers, and Griess reagent (SAN with 1-NA) was added at various times, decreasing amounts of Griess pigment were formed. The amount of nitrite free to form the pigment represents nitrite not reacted with the methoxy, amino, or methyl derivatives of aniline. The procedure, therefore, may be used to determine the rates of reaction of nitrite with these derivatives. The residual nitrite,

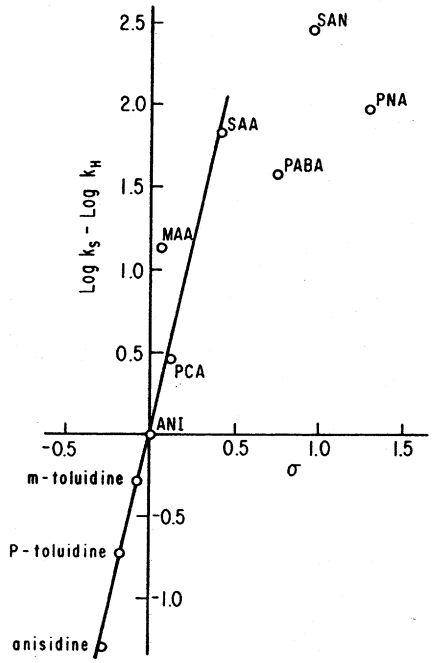

Figure 5. Rate of nitrosation or diazonium ion formation for various aniline derivatives as a function of the Hammett σ^- constant

plotted as a function of time, gave first-order curves which, in turn, gave nitrosation rate values of 0.014, 0.022, and 0.06 min^{-1} for *p*, *m*, and *o*-toluidine, respectively, and 0.008 for *p*-anisidine. The nitrosation of 1,4-benzenediamine was too fast to follow by the foregoing procedure; therefore, a mixture of 1.0 mM 1,4-benzenediamine and SAN with 0.2 mM 1-NA was reacted with nitrite, and the rate was calculated from the amount of interference in the nitrosation of SAN (Equation 7). The rate value so calculated was 1.74 min^{-1}. The aniline diazonium ion coupled with 1-NA at a very slow rate ($k_{1_{st}}$ = 0.0073 min^{-1}) to produce low absorption maxima at 525 and 390 (ϵ = 13.3 and 20.7 mM^{-1} cm^{-1}, respectively).

The results show the importance of strong negative inductance by the auxochrome on the ring structure, since –I substituents greatly increase the rate and amount of dye formed as compared to aniline, while +I compounds eliminate the dye formation. The rate controlling reaction, therefore, is the diazonium ion formation, especially since the nitrosation step, an electrophilic attack by N_2O_3, proceeds at near normal rates for the noncouplers.

Rate Constants and the Hammett Relationship. The rate constants for either nitrosation (*p*- and *m*-toluidine, and anisidine) or diazonium ion formation (PCA, MAA, SAA, PABA, SAN, PNA, and ANI—see Table I for definitions) are plotted as a function of the Hammett σ^- value in Figure 5. The σ^- values are specifically for the amino group and were obtained either from Johnson (*15*) or Hansch (*16*) (Dr. Hansch's group at Pomona College maintains a file of Hammett sigma values). The line is drawn based on the values for the nitrosation reaction because this is the reaction for which the σ^- value applies. Of the NS that also formed a diazonium ion, only three (PCA, ANI, and SAA) are on the line, implying that the internal diazonium ion formation is slower than the nitrosation. MAA appears to have too low a σ^- value and too low a normal σ value (see the results for the coupling reaction later in text). The σ^- value for MAA for this reaction probably should be 0.23. The faster reacting NS (PABA, SAN, and PNA) all react much slower than would be expected from their σ^- value, but, since the reaction is two-step, we assume that the internal diazonium ion formation is also rate limiting for these compounds. This assumption apparently leaves the three NS in a random array, yet there

Table II. First-Order Rate Constants and Hammett σ Values for Diazonium Ion Formation and the Coupling Reaction

coupling reagents	Nitrosated Species						
	PNA	SAN	SAA	MAA	PABA	PCA	ANI
1-NA k_1	0.21	0.34	0.18	0.09	0.14	0.046	0.029
(σ^-)	(1.27)	(0.94)	(0.40)	(0.23)	(0.73)	(0.11)	(0.00)
k_2	15.2	3.98	0.48	0.66	0.200	0.14	0.0073
(σ)	(0.76)	(0.57)	(0.42)	(0.40)	(0.27)	(0.22)	(0.00)
$k_1 \times k_2$	3.19	1.35	0.086	0.059	0.028	0.0064	--
NED[a]	15.2	3.22	1.13	1.69	0.295	0.100	
2SN	2.50	0.52	0.050	0.035	0.021	0.018	
5SN	0.54	0.105	0.010	0.010	0.008	0.005	
6SN	2.02	0.336	0.032	0.032	0.022	0.026	
7SN	5.24	1.06	0.055	0.041	0.055	0.030	
8SN	~0.0025[b]	0.272	0.040	0.005	0.040	0.012	
5ON	0.191	0.207	0.077	0.046	0.037	0.020	
7ON	0.174	0.33	0.155	0.063	0.092	0.028	
5AN	0.22	0.27	0.075	0.075	0.017	0.016	
5NN	0.33	0.090	0.011	0.020	0.040	0.0087	

[a] Rate constants for this and succeeding coupling reagents are for the second step reaction (k_2). [b] Zero-order reaction $k_0 = \mu M/min$.

is a rationale for the placement. The internal reaction to form the diazonium ion is initiated by an electron withdrawal, weakening the N to O bond. Since the $-SO_2R$ group is more electronegative than the other three groups (-Cl, $-NO_2$, -COOH) (*17*), the reactions would be expected to be much faster for the former group. If the data for the three sulfonic derivatives and the data for the other three are viewed as two separate sets, it is seen that they comprise two separate parallel lines, the difference between them being a measure of the increased electronegativity of the $-SO_2R$ group. The effect on the σ bond would be principally negative inductance (-I) hence the inclusion of MAA in the $-SO_2R$ curve, since the only effect of a group in the meta position is -I.

The coupling reaction rate constants for the coupling NS were plotted as a function of the normal σ value. With the exception of MAA, the plot was linear:

$$\log k_S - \log k_H = 4.42\ \sigma(\pm 0.36) + 0.23 \qquad r = 0.99,\ S_{yx} = 0.209 \quad (11)$$

where k_H and k_S are the first-order rate constants for aniline and its substituted derivative, respectively. As noted, metanilic acid reacted faster than expected. Zollinger (*18*) had observed that sulfanilic acid also reacted faster than expected and corrected the sigma value accordingly. By use of his value, the rate constant for SAA was on the curve of Equation 11. In view of the nitrosation/diazonium ion and coupling reaction data, we conclude that for these two reactions the previously reported σ^- and σ values for MAA are too low.

Second Step Rate Constants: Effect of Auxochrome on Naphthyl Derivatives. The second step rate constants are shown in Table II. With the exception of the coupling of NED with the three acidic auxochromes, SAA, MAA, and PABA, no substituents on any part of the 1-naphthylamine molecule increased the rate of the reaction. The degree of retardation of the reaction was least with substituents in the 7 position, and the kind of substituent was of little importance. A number of other CRs were studied which are not reported in Table II. The amino group in the 2 position of the naphthalene ring had a negative effect compared to the same group in the 1 position. 2-Naphthylamine and SAN reacted slower than 1-NA and SAN ($k_{1_{st}} = 0.69\ min^{-1}$ vs. $3.98\ min^{-1}$) to yield a final absorbance of 0.069 A.U. (10 μM nitrite). The 6-sulfonic derivative (6-amino-2-naphthalene sulfonic acid) reacted even slower ($k_{1_{st}} = 0.099\ min^{-1}$) and had about the same ΔA (0.073). The principal effect of the amino group is to increase the nucleophilic character of the para position on the one aromatic ring through a positive resonance (+R) effect. Substituents in the meta position do not exert resonance effects (R); hence the low reactivity of the 2-aminonaphthalene derivatives.

In contrast, substituents with either weaker +R effects or those that exert -R or -I effects resulted in slower reactions and lower amounts of pigment formed. The methyl group exerts a weak +R effect, but 1-methylnaphthalene reacted slowly ($k_{1_{st}} = 0.25\ min^{-1}$) to yield a ΔA of only 0.035. 1-Naphthol did not react at all. The acetic acid group exerts a -I effect, and *N*-1-naphthalene acetic acid did not couple, although converting the acid group to the acetamide resulted in a slow reaction for a low production of pigment ($k_{1_{st}} = 0.25\ min^{-1}$, $\Delta A = 0.040$). Comparing these results with the 2SN/SAN reaction, it is seen that in terms of rates and amounts (compare Table III), the effect of a relatively strong -I group in the meta position is relatively less important than having a -I group in the para position, since the 2SN reaction went faster to produce more pigment than did the *N*-acetic acid derivative.

Finally, the placement of a group in the para position (4 amino-1-naphthalene sulfonic acid) resulted in complete elimination of coupling. With both substituents on one ring, reaction with the other ring was possible, but did not occur. The results show that for reaction under these conditions there must be a relatively strong +R, +I substituent in the para position of one of the benzene rings of naphthalene.

These results are for the reaction in the range pH 2.75–2.85 and with SAN. Reactivities of many of the compounds are different at other pH values (*3*), particularly for the coupling reaction, and with other coupling reagents. For example, *p*-anisidine did not form a pigment with 1-NA but did do so with the more reactive NED.

λ and ϵ Values of the Absorption Maxima. An analysis of variance of Table III showed both NS and CR to be significant with respect to the position of λ_{max}, with the contribution from the CRs about twice that of the NSs. A number of CR and NS combinations produced multiple absorption maxima which, since the molecular structure involved would not be expected to have multiple resonance bands, probably indicate different pigment forms. The absorption coefficients at λ_{max}, which are the most important features of the pigments, did not increase with substituents on the naphthylamine ring. The maximum absorption values were not necessarily related either to the rate of the overall reaction or to either of the two steps in the reaction. While variations in intensity of absorption are to be expected within a class of optically absorbing compounds, it is possible that the extreme variations observed in Table III were not due to this cause, but were, in most cases, the result of incomplete conversion to pigment. By kinetic

Table III. λ_{max} and ϵ_{max} (mM^{-1} cm^{-1})

coupling reagents	Nitrosated Species: PNA λ	PNA ε	SAN λ	SAN ε	SAA λ	SAA ε	PABA λ	PABA ε	PCA λ	PCA ε	MAA λ	MAA ε
1-NA	520	40.6	519	39.5[a]	518	32.5[a]	526	36.0	530	21.3	520	28.5
NED	542	25.2	535	27.0	542	15.1	547	26.3	552	10.2	540	13.2
									402	8	397	6
2SN	527	20.9	513	13.0	540	9.7	520	17.2	~470	7	480	5.5
					410	6.8			560	3	520	5.2
5SN	518	22.5	511	21.9	521	13.0	518	18.7	~515	8.6	515	
					400	3						
6SN	519	34 U[c]	510	38.8	517	33.3	509	27.8	519	18.9 U[c]	518	28
7SN	514	34[b] VU[d]	511	40.0	520	36.3	512	33 VU[d]	520	17.7 VU[d]	518	32
							447	33				
8SN	520	31[b] VU[d]	514	38.2	524	36.8	521	34	527	16.3 U[c]	518	33
									390	(10)		
5ON	535	24.2	528	22.5	528	19.2	540	19	532	10	530	15
			408				420	5	405	4.0		
7ON	540	37 U[c]	533	33.0	540	27.2U	534	32	548	17.9	536	24.4
					412							
5AN	512	unstable	535	13.7	510	10.0	532	12	533		500	7
			460									
5NN	537	15.8	521	22.0	508	24	542	11	(530)	7	500	
							450	~13				

[a] Coefficient of variation = 2.5%. [b] Calculated from formula. [c] U = Unstable. [d] VU = Very unstable.

Table IV. ϵ_{max} for NED/1-NA and Various Nitrosated Species with and without Pre-Reaction with Nitrite

	NED: Pre-reaction none (% max)	NED: Pre-reaction NS + NO_2^-	1-NA: Pre-reaction none (% max)	1-NA: Pre-reaction NS + NO_2^-
SAN	27 (66)	41.0	39.5 (94)	42.0
SAA	15 (38)	39.8	32.5 (81)	40.0
PNA	25 (63)	39.6	40.6 (98)	41.6
PABA	26 (87)	30.0	36.0 (91)	39.4
PCA	10 (41)	24.2	21.3 (68)	31.2
MAA	13 (40)	32.9	28.5 (86)	33.0

analysis and previously described techniques we have been able to define and quantitate a number of the interfering reactions which result in less than maximal pigment formation.

Instability of Diazonium Ion. During the pre-reaction experiments it was observed that when the NSs were reacted with nitrite for extended periods, the total amount of pigment formed decreased with time, indicating a decomposition of the diazonium ion. The reaction was first order and was about 2–3%/h for SAN, PABA, PCA, and MAA. The reaction was negligible for SAA but amounted to about 18%/h for PNA. In the presence of reductants, diazonium ion reduction was highly significant in terms of pigment formed (see below).

Nitrosation of the Coupling Reagent. The values in Table III are the maximal values obtained by simultaneous addition of NS, CR, and nitrite. Higher final absorption values were obtained when the NS and nitrite were pre-reacted long enough for the NS to be converted to the diazonium ion (Table IV). We attribute the lower values in Tables III and IV to loss of nitrite by reaction with the CR. This conclusion would lead us to expect that the effect of pre-reaction would be less with the faster reacting NS. The value $k_1 \times k_2$ is a measure of the total reactivity (formation × intermediate reactivity) of the NS (Table II). Ordering the NS in terms of reactivity and % maximal reaction with 1-NA (Table IV), the following sequences were obtained:

NS reactivity = PNA > SAN > MAA > SAA > PABA > PCA

% max = PNA > SAN > PABA > MAA > SAA > PCA

As expected, with the exception of PABA, the faster the NS reacted, the lesser the effect of pre-reaction. PABA also showed less effect of pre-reaction with NED. Such an effect would be observed if PABA nitrosated readily, reducing the amount of free nitrite, but formed the diazonium salt at a slow rate. Conversely, the slower the CR coupled and nitrosated, the less would be the effect of pre-reaction. Thus the effect of pre-reaction was less for 1-NA (k = 0.045 min^{-1} for the nitrosation reaction) than for NED (k = 0.186 min^{-1}).

Nitrosation of the CR affects the dependence of the amount of pigment formed on the concentration of CR. At low concentrations of CR, with insufficient concentrations either for total conversion of the diazonium ion or for effective competition with other reactants for this ion, the amount of pigment will be low. Conversely, at high CR concentrations, appreciable amounts of nitrite react with the CR, and pigment formation is again reduced. The resultant effect is that there is a point of maximal pigment production with varying CR concentration. In practice, a range of maximal production was observed. For example, with the SAA concentration constant at 1.0 μM, the concentration of 1-NA was varied from 0.017 to 0.800 mM. Maximal production of pigment occurred from 0.033 to 0.200 mM; at 0.800, pigment production was 40% less. The rate of 1-NA nitrosation was 0.131 min^{-1}, determined by pre-reacting 1-NA and nitrite before adding SAN. With this value used in Equation 7a (with a large excess of CR it does not matter that the reactant removing nitrite from reaction with the NS is the CR), the theoretical reduction in pigment concentration with 0.800 mM 1-NA was calculated to be 27%, compared with the observed 40%.

It may be concluded that the low absorbance values in Table III are due in part to nitrosation of the CR, but not entirely, as seen in Table IV. Pre-reaction made little difference in the amount of pigment formed with PABA and NED, but even with pre-reaction, the final absorption attained was still low. While it is true that molar absorptivities vary from compound to compound, the values of 30 and below appear to be too low.

Effect of Varying the [NS] and [CR]. PNA, SAN, PABA, and PCA were chosen as representative of the range of reactivity of the NS and 1-NA, NED, and 7SN as representative of the group of CR. Pigment formation in a 0.010 mM solution of nitrite was measured at varying concentrations of the Griess reagents as shown in Table V. The center value

Table V. Effect of Varying the Relative Concentrations of the Nitrosatable Species and Coupling Reagent Final Absorption Values at λ_{max} for Griess Pigment[a]

coupling reagent	[mM]	Nitrosated Species: PNA 0.167	PNA 1.0	PNA 4.0	SAN 0.167	SAN 1.0	SAN 4.0	PABA 0.167	PABA 1.0	PABA 4.0	PCA 0.167	PCA 1.0	PCA 4.0
1-NA	0.033		0.405			0.415			0.335			0.274	
	0.20	0.291	0.410	0.405	0.324	0.421	0.430	0.301	0.360	0.354	0.104	0.232	0.314
	0.80		0.300			0.353			0.301			0.171	
NED	0.033		0.189			0.383			0.350			0.087	
	0.20	0.118	0.315	0.449	0.055	0.268	0.353	0.120	0.260	0.344	0.024	0.108	0.167
	0.80		0.146			0.087			0.135			0.041	
7SN	0.033		0.332 (f)			0.402			0.135			0.155	
	0.20	0.297 (s)	0.355 (m)	0.449 (f)	0.333	0.400	0.402	0.163 (s)	0.232 (s)	0.189 (s)	0.187	0.214 (m)	0.242 (m)
	0.80		0.320 (vs)			0.329			0.172			0.233	

[a] f = faded <2 min. m = faded <10 min. s = faded <1 h. vs = faded <6 h.

Figure 6. Formation and fading of the Griess pigment formed from *p*-nitroaniline and the four reactive sulfonic acid derivatives of 1-naphthylamine, 5SN, 6SN, 7SN, and 8SN. (For explanation of abbreviations, see Table I). --- □ 5SN, - - - △ 6SN, -·- ▽ 7SN, — ○ 8SN

in each combination is the amount of pigment formed from concentration of reagents as normally used for nitrite analysis (*13*). In some of the combinations the optimal concentrations, either relative or absolute, were not achieved. It cannot be assumed that the same optimal concentrations pertain to all reagent combinations, but must be individually determined.

The stability of the pigment once formed was affected by the reagents used. The PNA/7SN pigment faded at an increasing rate with increasing PNA concentration and a decreasing rate with increasing 7SN. A similar, but not as pronounced, effect was observed with the PCA/7SN pigment. Pigment fading with the other reagent combinations was so slow that it was not possible to positively demonstrate the effect, but pigment fading was faster at higher NS concentrations with a number of the NS studied, as indicated in Table III. "U" indicates a pigment that had a short period of relative stability, followed by a definitely observable increase in rate of fading. "VU" indicates a pigment that showed a sharp maximum, with the rapid fading phase following immediately after formation. The phenomenon is most pronounced for certain reagents and combinations and appears to be related to a number of factors of which concentration is but one. The reaction in air frequently was complicated by a slow increase in pigment after the initial rapid phase. This second phase probably was due to uptake of oxides of nitrogen from the air (*10*).

Mechanisms of Fading. Scans of the spectra of the pigment solutions during fading showed that the process occurred by conversion to either a colorless form or to some other pigment, the latter also being formed during the reaction. The first type of fading was characteristic of the PNA/sulfonated naphthylamine pigments, and the rate of fading was dependent on the position of the sulfonic group (Figure 6).

With the 7SN and 8SN pigments, fading began before formation had gone to completion. The first part of the reaction was first order (Equation 8), and by use of Equation 9 we were able to calculate a theoretical A_∞ value using absorbance rather than reagent concentrations (Table III) which was significantly higher than actually attained, i.e., fading began before full conversion to pigment.

Fading of the PABA/7SN pigment followed the second type of reaction, conversion to another pigment form (Figure 7). As the 515-nm absorption band decreased, the 448-nm band increased. As noted from the spectra recorded at maximal formation of the 515-nm peak, there was a shoulder at ~450 nm, indicating that some of the 448-nm absorbing material was already present. With other reagent combinations, some of the shorter wavelength bands appeared concomitantly with the 500–600 nm bands (Table III) and, in some cases, were stable. The shorter wavelength bands were not due to impurities for they did not appear consistently for all pigments formed from any one NS or CR. Thus PABA gave double and

Table VI. Effect of Ascorbate and NADH on the Formation of Pigment

	CR											
	1-NA						NED					
	ascorbate						ascorbate					
	simultaneous		pre-react[a]		NADH		simultaneous		pre-react[a]		NADH	
NS	0	+	0	+	0	+	0	+	0	+	0	+
SAN ΔA	0.371	0.209	0.421	0.237	0.370	0.240	0.251	0.189	0.410	0.270	0.236	0.191
%[b]	56		56		65		75		66		81	
SAA ΔA	0.348	0.260	0.396	0.278	0.320	0.225	0.156	0.199	0.365	0.316	0.166	0.147
%	75		70		70		128		84		89	
MAA ΔA	0.311	0.244	0.330	0.258			0.111	0.110	0.329	0.268		
%	78		78				100		81			
PNA ΔA	0.420	0.225	0.426	0.199			0.238	0.185	0.335	0.115		
%	53		47				78		34			
PCA ΔA	0.225	0.141	0.349	0.180			0.067	0.074	0.232	0.135		
%	63		52				109(?)[c]		58			
PABA ΔA	0.387	0.204	0.420	0.240			0.250	0.224	0.374	0.255		
%	57		58				90		68			

[a] Pre-react NS and nitrite. [b] Percent conversion to pigment with reductant as compared to conversion without reductant. [c] Total conversions too low to permit collection of accurate data, but amounts with and without ascorbate always about the same.

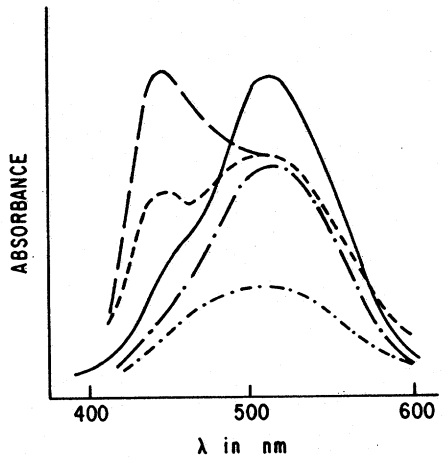

Figure 7. Spectral changes in the pigment formed from PABA, 7SN and nitrite — maximal formation of 515-nm absorption maximum. --- intermediate spectra later. -- maximal formation of 448-nm maximum. -·- maximal absorption with added ascorbate. -·- Previous reaction after pigment fading

triple absorption bands with only a few NS; 8SN produced multiple bands with only a few CR.

The fading reaction was primarily an oxidative process. Exclusion of air slowed but did not eliminate fading. The addition of ascorbate to the PABA/7SN reaction reduced the total amount of 515-nm pigment formed and almost totally eliminated the 448-nm band. Similarly, when ascorbate was added to the PNA/7SN/NO_2^- system simultaneously, the total amount of pigment formed was reduced, but what was formed was much more stable. However, the addition of ascorbate at the time of maximal pigment production had no effect on the rate of fading. The removal of oxygen by direct reaction with ascorbate is not fast enough to account for the stability of the pigment in either experiment. The reaction of nitrite with ascorbate could do so, however, Nitric oxide, produced from the reduction of nitrite, reacts swiftly with oxygen to re-form nitrite, and it was probably this cycle, operating while there was still free nitrite in the solution, that removed the oxygen.

Effect of Reductants. Ascorbate, cysteine, and NADH were added to selected reagent combinations to determine their effect on the reaction system. Reductants a priori may be expected to react with either the nitrite or the diazonium ion, with possibly some effect on the stability of the pigment. All of these reactions were of importance with respect to some combinations of reagents. Table VI summarizes the effect observed for ascorbate and NADH; under these conditions cysteine reduced the amount of pigment formed, but the effect was uniform. When all the nitrite was converted to the diazonium ion, the reduced amount of pigment formed with 1-NA was the same as when the reagents were all added simultaneously. If the nitrite–reductant reaction were to remove any nitrite permanently from the reaction, simultaneous addition would have resulted in less pigment. Since it did not, we conclude that the nitrite–reductant–oxidant cycle is not a factor in total pigment production. The diazonium ion/reductant reaction is, therefore, the major interfering reaction. The orders of relative interference in the coupling reactions and coupling rate constants (Table II) are:

CR	factor	relative reactivity
I-NA	interference	PNA, PABA, SAN, PCA > SAA, MAA
	coupling k	PNA >> SAN > MAA > SAA > PABA > PCA
NED	interference	PNA. SAN > PCA, PABA > MAA > SAA
	coupling k	PNA >> SAN > MAA > SAA > PABA > PCA

In contrast to the interference by CR nitrosation, the ordering of interference by ascorbate is not well correlated with diazonium ion reactivity. A close correlation is not necessarily expected, since the two reactions are not the same types. The reductant/diazonium ion reactions are electron transfers, whereas coupling is an electron sharing reaction.

The effects of reductants are not limited to diazonium ion reduction. The decrease in pigment produced when ascorbate was added was less with NED than with 1-NA. With the NED/SAA combination, and possibly NED/PCA, there was an increase in the total amount of pigment formed with ascorbate, but not with NADH. A study of the mechanism of the formation of nitrosylmyoglobin (*19, 20*) showed that ascorbate forms a semistable nitroso intermediate, which would lead to a reaction mechanism as shown in Equation 5b. As in the formation of the nitrososulfonic group, the nitrosoreductant intermediate removes a certain amount of nitrite from the reaction, then releases it slowly. As expected from this type of reaction, the reaction curves were biphasic (compare Figure 4). The initial phase of the reaction varied with reagent combination, but the rate constant of the second phase was always the same, $k = 0.16\ min^{-1}$. A second log phase

was not observed with added NADH, but the nitroso-NAD intermediate may not be as stable as the nitrosoascorbate intermediate. If the nitroso reductant were to react preferentially with the NS rather than the CR, the net result would be an increase in Griess pigment.

Effect of pH. A few selected reagent combinations were reacted at varying pH values. All showed maximal pigment formation from about pH 2.5–3.5, confirming a previous report (*7*). Since the pK_a values for the reagents varied, we assume the single range is due to protonation of nitrite to nitrous acid, $pK_a = 3.4$, which in turn forms the nitrosating species N_2O_3 (or in HCl, NOCl). The range represents a balance between too little HNO_2 at higher pH values, and protonation of the naphthalene derivative and/or acid decomposition of the pigment at lower pH values.

Effect of Temperature. SAN/1-NA and SAA/NED were reacted at 10, 20, 30, and 40 °C ± 1 °C with ΔH_a values of 40.6 and 30.2 Kcal/mol, respectively. These values are for the diazonium ion formation which is rate controlling for the overall reaction and may be compared to previously reported values of ~15 Kcal/mol for the coupling reaction (*21*). Final absorption values were maximal at 20 and 30 °C, with about 30–40% decrease in pigment formed with both reagent pairs at the highest and lowest temperatures. Therefore, with no need for temperature control, the reactions are best carried out at room temperature.

DISCUSSION

Sources of Variability. As a secondary result of this study, we have developed some data as to the sources of variability in Griess analysis. Griess reagents deteriorate on standing, even in the refrigerator, so we ran standard reactions to make sure reagent deterioration was within acceptable limits. With fresh solutions, the cumulative coefficient of variation was ±2.5%. Standard dilution curves had coefficients of variation (at 10 μM NO_2^-) about 1%, and in a study of the AOAC method of nitrite analysis (*9*) we obtained an average coefficient of variation of 1.5%. In contrast, collaborative studies have shown inter-laboratory coefficients of variation of 9% (*22*) and 11% (*13*) for residual nitrite in cured meat products. In view of the complex nature of both muscle tissue and the Griess reaction, the magnitude of the difference in coefficients of variation between what could be expected, 1–2%, and what is observed 9–11%, is not unexpected and may be irreducible.

Effect of pH. Little work has been done on the diazotization reaction in the pH region around 2.75–3.00, most studies being carried out in perchloric acid solution stronger than 10^{-2} M (pH 2.0) (*23*). The differences in reactivity of the protonated and free amine toward the nitrosating species is highly critical, since the pK_a values of the NS lie in this pH region. Thus, PNA and SAN were unprotonated, PABA and SAA were partially ionized, and PCA and MAA were essentially fully (>95%) ionized. It is generally accepted that the reaction is faster with the free amine, although the difference in reaction rate between free and protonated amine is not great (*23*) and in some situations is reversed (*24*). Conversely, electron withdrawing substituents ($-SO_2H$, $-NO_2$) normally decrease the rate of nitrosation (*23*). Finally, in reactions of equimolar nitrite and NS at about pH 2.0, the reaction depends on the rate of nitrous anhydride (N_2O_3, NOX) formation. Since the Griess nitrite analysis reaction is carried out with large reagent excess (100× NS), the rate of the nitrosation reaction would be expected to be totally nitrite dependent, that is independent of both NS concentration and structure. As observed, it was not, even though the nitrosation rates for all NS were governed by the nitrous acid concentrations. These results are consistent with the presently accepted reaction mechanism (*23*):

$$RNH_2 + N_2O_3 \overset{I}{\rightleftharpoons} RN^+H_2NO \rightleftharpoons (I)n \rightarrow P \quad (12)$$

where (I)n represents a series of reaction intermediates and P a product not in equilibrium with its precursors. The concentration of all intermediates is determined by the concentration of the nitrosating species which in turn is governed by its rate of formation from nitrous acid and the equilibrium steps, hence the pH dependence. The actual rate of disappearance, however, must be governed by the shift to the right, which is a function of the chemical characteristics of all of the individual NS intermediates. Since the NS is in large molar excess, equilibrium I is driven to the right, so that the subsequent shift to the right goes at a maximal rate only slightly affected by the NS concentration, as observed (relatively broad range of maximal rate of formation of pigment with varying [NS]). Since nitrosation, as represented by the equilibrium steps of the left hand side of Equation 12, precedes, and therefore must be faster than, the formation of products, the falloff of the coupling NS from the nitrosation curve in the Hammett plot is expected, since we were measuring only the formation of product, the diazonium ion.

On the other hand, pH does have a reagent effect in the coupling reaction. Zollinger (*18*) reported that the methyl and methoxy derivatives of aniline will couple, albeit very slowly. Although he does not say so, it is presumed that he used the maximal coupling rates attainable, since he reported using buffers of varying pH for his reactions. Because we were interested in the reaction only under relatively restricted conditions (pH 2.75) we did not determine the maximal rates attainable for the various coupling reagents. In this restricted range, not all NS or CR will couple. The lack of detectable pigment formation with the methyl or methoxy derivatives under these conditions probably reflects lowered reactivity of the CR (1-NA) at pH 2.75.

Reactivity and Utility. Reactivity of any given NS at pH 2.75 may be predicted from its Hammett constant, but the rate of reaction of a given nitrosated species or coupling reagent is not necessarily related to its usefulness for nitrite analysis. Because of the many extraneous reactions that take place, slowly reacting reagents are clearly not indicated. Conversely, PNA and NED were the fastest reacting NS and CR, but, as has been shown in this paper, neither is a reagent of choice for the reaction. Sulfanilamide, which forms a diazonium ion and couples slower than PNA, is a superior reagent since it forms stable pigments. In terms of rates, SAA is not too inferior to SAN, yet it gives lower final absorption values, which raises some question as to whether complete conversion has taken place. As a general rule, where there is a question of completeness of conversion, a reagent should not be used unless the nature of the side reaction is understood and controllable. Similar arguments may be made with respect to the coupling reagents. NED is a rapid coupler but, because it nitrosates rapidly, is not a good reagent for the reaction. 1-NA was the best CR studied in terms of rapidity, conversion, freedom from extraneous reactions, and pigment stability Unfortunately, it has been placed on the OSHA list of suspected carcinogens (*25*), and other compounds have been suggested as replacements (*13*, *26*). Normally, the substitution of one reagent for another should be on the basis of improving the procedure. The results in Table III indicate that substituted naphthylamines are not as good as 1-NA, yet 6SN, 7SN, or 8SN with SAA or SAN are acceptable.

The results of this study show that the amount of diazo pigment formed from the reaction of a variety of aniline and naphthylamine derivatives with nitrite is dependent on a number of factors, namely:

1. Kind and concentration of reagents used, including position of ring substituents.

2. Specific combinations and relative concentrations of nitrosatable species and coupling reagents.
3. Reaction of nitrite with ring substituents other than the amino group.
4. Reaction of nitrite with the coupling reagent.
5. Formation of more than one pigment.
6. Oxidation of the diazonium ion intermediate.
7. Oxidation of the pigment.
8. Oxides of nitrogen in the air.
9. Reduction of the diazonium ion by residual reductants.
10. Formation of semistable nitroso-reductant intermediates.
11. Pre-reaction of NS and nitrite.

These factors, in addition to pH and temperature, are important in some degree to all reagents studied and, it may be assumed, to any other compounds that have been or may be proposed for the purpose of determining nitrite. We know of no papers on nitrite determination where new or different reagents have been proposed in which more than one of these factors has been investigated. Since the operation of many of these factors results in significantly different total amounts of pigment formed, no new reagents or combinations thereof should be recommended without an assessment of the effects of these factors. This study also provides information that could be useful in improving the method of analysis.

In a study of a number of sample preparation procedures (*4*), we found that the different procedures yielded different amounts of nitrite. It is axiomatic that measured nitrite is a function of both sample preparation and colorimetric development, with interactions occurring between the two. The next step in improving the determination of nitrite by Griess reagents would be to study these interactions using the observations made in this study to determine those sample components affected by a given preparation procedure. For example, the difference in the effects of reductants on different colorimetric reagent combinations could be used to define the nature of residual reductants in cured meats and the effectiveness of sample preparation procedures in removing them.

The foregoing factors are not the entirety of extraneous factors or reactions that are of concern in nitrite determination. Nitrite itself is highly reactive in acid solutions. In addition to the bimolecular reaction to form the nitrosating compound, N_2O_3, nitrous acid undergoes a termolecular reaction to form NO and NO_3^-. Changes in concentration of nitrite and the pH will therefore affect the relative rates of these two reactions. Only under identical conditions can inhomogeneous multiple reactions give identical yields of a given product. In view of the many possible reactions involved in the Griess reaction, the importance of control of the reaction conditions is readily apparent.

LITERATURE CITED

(1) J. P. Griess, *Philos. Trans. R. S. (London)* 679 (1864).
(2) J. P. Griess, *Ber. Dtsch. Chem. Ges.*, **12**, 426 (1879).
(3) J. M. Tedder, *Chem. Synth. Dyes*, **3**, 223 (1970).
(4) R. N. Fiddler and J. B. Fox, Jr., *J. Assoc. Off. Anal. Chem.*, **61**, 1063 (1978).
(5) E. Sawicki, T. W. Stanley, J. Pfaff, and A. D'Amico, *Talanta*, **10**, 641 (1963).
(6) M. L. Ilosvay, *Bull. Soc. Chim.*, **2**, 388 (1889).
(7) B. F. Rider, *Ind. Eng. Chem.*, **18**, 96 (1946).
(8) G. Lunge and A. Lwoff, *Z. Angew. Chem.*, **7**, 348 (1894).
(9) R. N. Nicholas and J. B. Fox, Jr., *J. Assoc. Off. Anal. Chem.*, **56**, 922 (1973).
(10) Berl-Lunge, "Chemisch-technische Untersuchungsmethoden", E. Berl, Ed., II, 1, 8th ed., Julius Springer, Berlin. Photolith reproduction, Edwards Bros., Ann Arbor, Mich., 1943.
(11) L. Kamm, D. F. Bray, and D. E. Coffin, *J. Assoc. Off. Anal. Chem.*, **51**, 140 (1968).
(12) H. Erlbach, *Chem. Ber.*, **68**, 534 (1935).
(13) R. N. Fiddler, *J. Assoc. Off. Anal. Chem.*, **60**, 594 (1977).
(14) E. A. Guggenheim, *Philos. Mag.*, **2**, 538 (1931).
(15) C. D. Johnson, "The Hammett Equation", Cambridge University Press, London, 1973.
(16) C. Hansch, personal communication, 1977.
(17) J. Hine, "Physical Organic Chemistry", McGraw-Hill, New York, 1956.
(18) H. Zollinger, *Helv. Chim. Acta*, **36**, 1730 (1953).
(19) J. B. Fox, Jr., and J. Thomson, *Biochemistry*, **2**, 465 (1963).
(20) J. B. Fox, Jr., and S. A. Ackerman, *J. Food Sci.*, **33**, 364 (1968).
(21) J. B. Conant and W. D. Peterson, *J. Am. Chem. Soc.*, **52**, 1220 (1930).
(22) W. Landmann and M. C. Worland, *J. Assoc. Off. Anal. Chem.*, **47**, 395 (1964).
(23) A. F. Hegarty, in "The chemistry of diazonium and diazo groups", S. Patai, Ed., John Wiley & Sons, New York, 1978, p 511.
(24) E. C. R. de Fabrizio, E. Kalatzis, and J. H. Ridd, *J. Chem. Soc. B*, **533** (1966).
(25) OSHA, CFR 20:1910, 1976.
(26) R. Hilsheimer and J. Harwig, *Can. Inst. Food Sci. Technol. J.*, **9**, 225 (1976).

RECEIVED for review February 2, 1979. Accepted May 7, 1979.